

Echinacea alkylamides inhibit interleukin-2 production by Jurkat T cells

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Abstract:

Alkylamides present in *Echinacea* species have reported immunomodulatory actions, yet their direct effects on T lymphocytes, key mediators of antiviral immunity, are poorly understood. We hypothesized that constituents present in ethanolic extracts of *Echinacea* species exert direct immunomodulatory effects on human Jurkat T cells. Modulation of IL-2 production by submaximally stimulated Jurkat cells was determined in response to treatment with extracts prepared from dried aerial parts of *Echinacea purpurea*. Cells were treated with the extracts, with alkylamides or caffeic acid derivatives isolated from *Echinacea* species, or with corresponding ethanol vehicle, in the absence or presence of phytohemagglutinin and phorbol ester. *E. purpurea* extracted in a solvent mixture of 95:5 ethanol/water dose-dependently inhibited IL-2 production. This IL-2 inhibitory activity correlated with the presence of alkylamides but not caffeic acid derivatives, as determined by high performance liquid chromatography/electrospray ionization-mass spectrometry analysis. Simultaneous measurement of secreted IL-2 by ELISA and cell viability by the XTT assay showed that the 95:5 ethanol/water extract of *E. purpurea* was both IL-2 suppressive and cytotoxic at 50 and 100 µg/mL. Lower concentrations from 6.25 to 25 µg/mL significantly decreased IL-2 production but not cell viability. Alkylamides at concentrations found in a 50 µg/mL extract decreased IL-2 production by approximately 50%. Two *Echinacea*-derived alkylamides significantly depressed IL-2 production but not cell viability in a dose-dependent manner. Thus, alkylamides present in *E. purpurea* suppress the ability of activated Jurkat T cells to produce IL-2 independently of direct, cytotoxic effects.

Keywords: Alkylamides | Echinacea | Interleukin-2 (IL-2) | Jurkat T cells

Article:

1. Introduction

Perennial plants in the genus *Echinacea* have been traditionally used by Native Americans to treat cold and flu symptoms, reduce inflammation and heal wounds^[1]. One of the main constituent groups present in ethanol/water extracts of *Echinacea* with reported bioactivity is the alkylamides. Reported activities of *Echinacea* alkylamides include both anti-inflammatory and macrophage modulatory effects. Dose-dependent inhibition of 5-lipoxygenase by an alkylamide fraction of *Echinacea purpurea* roots (92.5% at 50 μ M) has been reported^[2]. Specific *Echinacea* alkylamides have since been identified that inhibit both 5-lipoxygenase and cyclooxygenase, upstream enzymes involved in the production of leukotrienes and prostaglandins^{[3] and [4]}. Evidence that alkylamides from *Echinacea* induce tumor necrosis factor α (TNF- α) mRNA but not protein in LPS-treated primary human monocyte/macrophages has been reported in vitro^[5], as has their inhibition of LPS-induced NO production in human macrophages^[6]. In vivo, 4-day oral administration of 12 μ g/kg/day *E. purpurea* alkylamides significantly enhanced phagocytic activity of alveolar macrophages in mice^[7]. Caffeic acid derivatives also present in *Echinacea* extracts are reported to have direct antiviral activity^{[8] and [9]}. These data suggest that alkylamides have the dual actions of anti-inflammatory and indirect antiviral effects, which together may influence the course of upper respiratory infection (URI)^[10]. However, no data on the effects of alkylamides on measures of T cell-specific immunity, known to play a key role in cell mediated immune responses to URI^{[11] and [12]} have been reported.

Few studies report the modulation of T cell activation markers, such as IL-2, by *Echinacea*. IL-2 is a critical autocrine growth factor upregulated upon T cell activation that is required for the clonal expansion of T cells^[13]. IL-2 is also an important cytokine involved in regulatory T cell activation and expansion^[14]. The production of IL-2 has been linked to decrease in symptoms and reduction of virus recovery in experimental rhinovirus infection^[15]. Thus, modulatory effects on IL-2 protein production may influence T cell specific immune responses to infection. In vivo, 4 weeks of oral administration of *Echinacea* aerial parts increased serum IL-2 levels 320-fold in rats^[16]. In vitro, IL-2 mRNA production was decreased over a 24-h period in human peripheral blood leukocytes treated with an *E. purpurea* fresh plant extract^[5]. Neither of these studies assessed the effects of *Echinacea* on IL-2 induction by activated T cells. Thus, the effects of *Echinacea* and alkylamides on T cell activation-induced IL-2 production are unknown. To test the hypothesis that *Echinacea* extracts modulate mitogen-induced IL-2 secretion, we assessed the effects of four aerial *E. purpurea* extracts and two classes of reportedly bioactive constituents, alkylamides and caffeic acid derivatives, on IL-2 production and cell viability upon PHA/PMA stimulation of Jurkat E6.1 T cells.

2. Material and methods

2.1. *Echinacea* extracts

Extracts were prepared at HerbPharm, Inc. (Williams, OR) from 2-year-old cultivated *E. purpurea* leaves and flowers by drying at 80 °C for 8 days and macerating for 7 days at a starting ratio of 1:5 (w/v) in four different ethanol/water menstruums (95:5, 75:25, 50:50, and 25:75). A voucher specimen was independently species verified (Steven Foster Group, Inc., Fayetteville, Arkansas). Undiluted extracts tested negative for microbial contamination by triple sugar iron plate inoculation. Extracts tested negative for endotoxin contamination by the Limulus Amebocyte Lysate (LAL) gel assay (Cambrex Corp., East Rutherford, NJ) at the limit of detection (< 0.125 EU/mL). For testing, dry plant masses were measured after lyophilization, and mg dry plant material per mL diluent determined. *E. purpurea* extracts were diluted to 0.1 to 100 µg dry plant material/mL final concentration for assay of IL-2 production alone, and diluted to 1.25 to 100 µg/mL for the IL-2 production/cell viability experiments, with ethanol adjusted to 0.5% final concentration.

2.2. *Echinacea* reference standards

Reference standards of *Echinacea* spp. (ChromaDex, Inc., Santa Ana, CA), known to be present in *E. purpurea* extracts, included:

Alkylamides:

- 1) Dodeca-2(*E*),4(*E*),8(*Z*),10(*Z*)-tetraenoic acid isobutylamide;
- 2) Dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide

Caffeic acid derivatives: (1) Caftaric acid; (2) Cichoric acid; (3) Chlorogenic acid

Alkylamides and cichoric acid were reconstituted in 95% ethanol, and caftaric acid and chlorogenic acid in 47.5% ethanol (in distilled water) to prepare 5 mg/mL stock solutions. Reconstituted stock solutions were diluted to 0.625–25 µg/mL final concentration for assay. The 25-µg/mL standard solutions tested negative for endotoxin contamination at the limit of detection (< 0.125 EU/mL).

2.3. Mitogenic stimulation

Optimal stimulant conditions and incubation time to elicit sub-maximal IL-2 production by Jurkat cells were determined using phytohemagglutinin (PHA, Sigma-Aldrich, Milwaukee, WI) and phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich). Jurkat cells (1×10^6 cells in 1 mL) were treated with 5 µg/mL PHA; 10 ng/mL PMA; or 1 µg/mL PHA + 1 ng/mL PMA and incubated at 37 °C, 5% CO₂, and 95% relative humidity. Supernatants were harvested after 4, 12, and 24 h and stored at – 80 °C until IL-2 ELISA (IL-2 DuoSet, R&D Systems Minneapolis, MN). Optimal conditions for sub-maximal IL-2 production in the IL-2 production/cell viability

assay were determined by titrating PHA concentrations at a fixed PMA concentration (1 ng/mL) at four different cell concentrations from 5×10^3 – 4×10^4 cells/mL. Secreted IL-2 concentration vs. PHA concentration was plotted and IL-2 concentration determined by the association curve produced using LABfit® Curve Fitting Software v.7.2.33 ^[17].

2.4. Cell culture followed by ELISA

Jurkat E6.1 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 containing phenol red, 25 mM HEPES, 1 mM sodium pyruvate, 2 mM l-glutamine and 10% fetal bovine serum (Life Technologies, Inc., Rockville, MD) and plated at 1×10^6 cells in 1 mL final volume. Cells were either untreated or treated with *E. purpurea* extracts (0, 0.1, 1, 10, 50, and 100 µg/mL in 0.5% ethanol final concentration) and either unstimulated or stimulated with PHA (2.5 or 5 µg/mL) and PMA (1 ng/mL). Supernatants from single wells per test condition were collected at 24 h and stored at – 80 °C until IL-2 ELISA. IL-2 levels of triplicate samples were determined by subtracting background absorbance at 550 nm (OD₅₅₀) from absorbance at 450 nm (OD₄₅₀) using a microplate reader (FL-600, Bio-Tek, Inc., Woburn, MA) and extrapolation from IL-2 standard curves using accompanying KC4 software (Bio-Tek, Inc., Woburn, MA).

2.5. Simultaneous IL-2 production/cell viability assay

An assay was developed to measure cell viability and IL-2 concentration of supernatants from the same wells following treatments. Medium as described in Section 2.4 but without phenol red was used. Triplicate cultures for each test condition served as three independent measurements of IL-2 production and cell viability. Jurkat cells were plated in 96-well plates at 2×10^4 cells/well in 200 µL. For the cell viability standard curve, cells plated in triplicate wells at 2.5×10^4 ; 2×10^4 ; 1.5×10^4 ; 7.5×10^3 ; 5.0×10^3 ; 2.5×10^3 ; and 0 cells/well (in 250 µL) were unstimulated. Test samples (25 µL *E. purpurea* (95:5 ethanol/water), 0.5% ethanol vehicle control, or standard isolates) and 25 µL stimuli (6 µg/mL PHA + 1 ng/mL PMA final concentration) were added. After 24 h at 37 °C, 5% CO₂, 95% relative humidity, supernatant (100 µL) was removed without disturbing cells and tested for IL-2 using ELISA.

For the XTT/PMS assay to assess cell viability ^[18], 100 µL of supernatant was removed from cell standard wells, and 100 µL of 1 mg/mL 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma-Aldrich) plus 0.02 mM phenazine methosulfate (PMS, Sigma-Aldrich) in medium added. After 5 h at 37 °C, 5% CO₂ and 95% humidity, OD₄₅₀–OD₆₅₀ was measured and a cell concentration standard curve generated, from which viable cell numbers of the test wells were extrapolated. The caffeic acid derivatives reacted with XTT/PMS, adding color that interfered with absorbance readings. Interference was compensated for by creating no cell control wells of the XTT/PMS reagent and caffeic acid derivatives at each test concentration, and subtracting calculated cell number of corresponding control well from sample cell number.

2.6. Analysis of caffeic acid derivatives and alkylamides with HPLC/ESI-MS

The caffeic acid derivatives and alkylamides in the *E. purpurea* extracts were analyzed using high performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI-MS). For each sample, an aliquot (1 mL) of *E. purpurea* extract was centrifuged at 200×g to remove particulate matter and filtered with a 0.2-μm nylon filter. A binary pump HPLC (HP1100, Agilent, Palo Alto, CA) with a C18 column (Alltech, 50 × 2.1 mm, 3.0 μM particle size) was used, and duplicate injections of 10 μL performed. The gradients employed were (A = 1% acetic acid in nanopure water and B = HPLC grade acetonitrile):

1) Caffeic acid derivatives: $t = 0$ to 4 min, 90% A; $t = 4$ to 15 min, 90% A to 60% A; $t = 15.1$ to 19 min, 0% A; $t = 19.1$ to 24 min, 90% A.

2) Alkylamides: $t = 0$ to 4 min, 50% A; $t = 4$ to 11 min, 50% A to 23% A; $t = 11.1$ min to 16 min, 0% A; $t = 16.1$ to 19 min, 50% A.

The HPLC was interfaced to an ion trap mass spectrometer with an electrospray ionization source (LCQ Advantage, Thermo Finnigan, San Jose, CA) for detection. Caffeic acid derivatives were analyzed in the negative ion mode and alkylamides in the positive ion mode. The scan range was 150 to 1500 m/z , capillary temperature was 275 °C, and spray, capillary, and tube lens voltages were 4.5 kV, 10 V, and 50 V, respectively.

Constituents in the extracts were identified according to their molecular weights, HPLC retention times, and MS–MS fragmentation patterns using a previously published method^[19]. Quantification was accomplished as described^[19]. Standards (Chromadex, Santa Ana, CA) were used to quantify caffeic acid derivatives, and alkylamide concentrations were estimated based on a calibration curve for undeca-2Z,4E-diene-8,10-diyonic acid isobutylamide, which was isolated from an *E. purpurea* extract.

2.7. Statistical calculations

Data were subjected to one-way ANOVA using SPSS 13.0 (SPSS Inc., Chicago, IL) with each extract or constituent as a factor and each concentration as a different treatment. The treatment means ($N = 3$) were compared to vehicle control using Tukey's Honestly Significant Different (HSD) post hoc analysis (two-tail, alpha level 0.01).

3. Results

3.1. PHA titration to determine submaximal IL-2 production

Jurkat cell stimulants were selected based on earlier published studies^[20] and confirmatory experiments. PHA or PMA alone did not stimulate IL-2 production over 24 h, but the combination did. The optimal mitogenic stimulation conditions for sub-maximal IL-2 production in the IL-2 production/cell viability assay were determined in PHA titration experiments, with PMA held constant at 1 ng/mL. For 2×10^4 cells/well, the PHA concentration yielding approximately half maximal IL-2 concentration (714 pg/mL) was calculated to be 6 μg/mL PHA

(Fig. 1). A confirmatory ELISA of the same supernatant showed 654 ± 50 pg/mL IL-2 induced by 6 $\mu\text{g/mL}$ PHA and 1 ng/mL PMA, verifying these conditions as appropriate for the IL-2 production/cell viability assay.

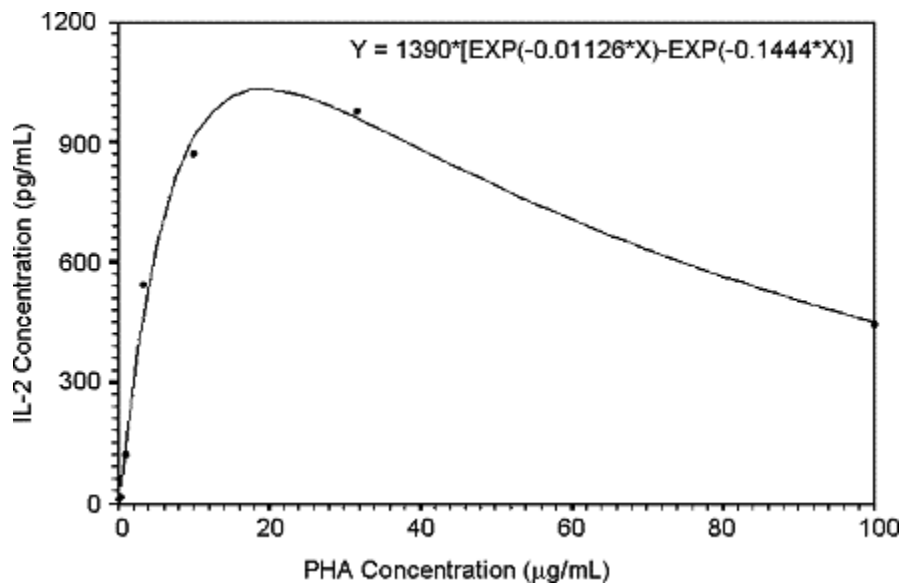


Fig. 1. IL-2 response of Jurkat T cells to increasing PHA and fixed PMA concentrations. PHA concentrations were tested at 1/2 log dilutions from 0.1 to 100 $\mu\text{g/mL}$. The association curve was extrapolated by specifying the maximum of 4-point parameters with the lowest chi-square value for the best fit. Maximum IL-2 at 24 h was calculated to be 1032.9 pg/mL at the PHA concentration of 19.2 $\mu\text{g/mL}$ and 714.7 pg/mL at 6 $\mu\text{g/mL}$ PHA, with cell concentration at 2×10^4 cells/well.

3.2. *E. purpurea* extracts suppressed IL-2 production

Echinacea treatment alone did not stimulate IL-2 production (data not shown). The effect of treatment with four distinct *E. purpurea* extracts (95:5, 75:25, 50:50, or 25:75 ethanol/water menstruums) on IL-2 production upon PHA/PMA-stimulation of Jurkat cells was determined (Fig. 2). Mean percent IL-2 production relative to the stimuli alone condition and standard deviations from three independent experiments are shown. *E. purpurea* extracted in 95:5 ethanol/water suppressed IL-2 production by approximately 65% at 50 $\mu\text{g/mL}$, compared to 0.5% ethanol vehicle-treated cells. This IL-2 suppressive effect was statistically significant ($p < 0.0001$) at 50 and 100 μg plant material/mL.

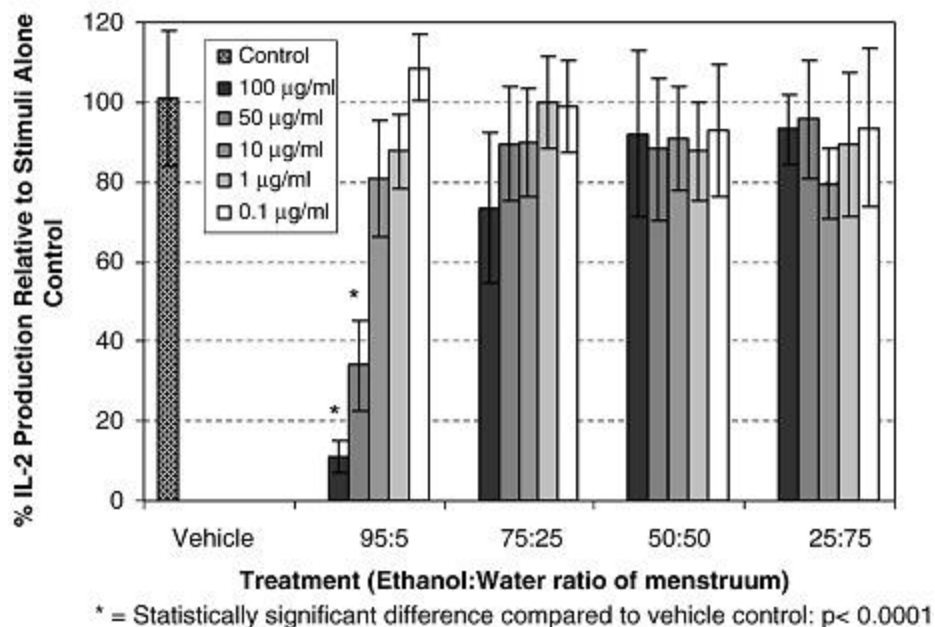


Fig. 2. IL-2 response of PHA/PMA stimulated Jurkat T cells to *E. purpurea* extracts. PHA/PMA stimulated Jurkat T cells were incubated with stimuli alone, vehicle (0.5% ethanol), or increasing concentrations of extracts, prepared from four distinct solvent mixtures: 95:5; 75:25; 50:50; and 25:75 (ethanol/water). For each treatment condition, the ratio of measured IL-2 level was divided by the mean of stimuli alone control. Error bars represent the variability of three independent assays. Final ethanol concentration of each extract on cells was adjusted to 0.5%. The treatment means ($N = 3$) were compared to vehicle control using Tukey's Honestly Significant Difference (HSD) post hoc analysis (two-tail, alpha level 0.01).

3.3. Alkylamides and caffeic acid derivative content varied in distinct *E. purpurea* extracts

The alkylamides and caffeic acid derivatives detected in *E. purpurea* extracts are known constituents of this plant, and their structures have been published elsewhere^[21]. The most abundant alkylamides were dodeca-2(*E*),4(*E*),8(*Z*),10(*E/Z*) tetraenoic acid isobutylamide and undeca-2(*Z*),4(*E*)-diene-8,10-diynoic acid isobutylamide. Table 1 indicates the amount of each compound found in the original, undiluted *E. purpurea* extracts. Alkylamides were extracted at the highest level by 95:5 ethanol/water (964 µg/mL), while no alkylamides were present in the 25:75 ethanol/water extract. In contrast, the highest amount of cichoric acid (1030 µg/mL) was found in the 50:50 extract, with much less in the 95:5 extract (13 µg/mL).

Table 1. Constituent concentrations in original *E. purpurea* extracts

<i>E. purpurea</i> constituent	95:5 ^a (26.7 mg/mL)	75:25 ^a (85.3 mg/mL)	50:50 ^a (93.0 mg/mL)	25:75 ^a (95.3 mg/mL)

	Concentration \pm absolute standard deviation in $\mu\text{g/mL}$			
Undeca-diene-diynoic acid isobutylamide	24 ± 1	6 ± 1	4 ± 1	0 ± 0
Dodeca-tetraenoic acid isobutylamide	940 ± 30	720 ± 20	215 ± 6	0 ± 0
Caftaric acid	8 ± 1	112 ± 2	461 ± 5	422 ± 5
Cichoric acid	13 ± 7	580 ± 20	1030 ± 40	660 ± 20
Chlorogenic acid	4 ± 6	19 ± 6	12 ± 6	4 ± 5
Total alkylamides	964 ± 30	726 ± 20	219 ± 6	0 ± 0
Total caffeic acid derivatives	25 ± 9	711 ± 20	1503 ± 40	1086 ± 30

Undiluted *E. purpurea* extracts prepared with solvents of different ethanol/water ratios were analyzed by HPLC/ESI-MS. Caffeic acid derivatives (caftaric, cichoric, chlorogenic acid) and alkylamides were eluted by different HPLC water/acetonitrile gradients. Quantification of caffeic acid derivatives was performed using commercially available standards and alkylamides were quantified using an isobutylamide isolated from an *E. purpurea* extract. The constituent profiles differed considerably depending on the ethanol content used in the extraction solvent.

^aEthanol/water ratio of solvent mixtures used to prepare each *E. purpurea* extract.

Concentrations in parentheses indicate dry plant material/mL solvent.

Because the 95:5 ethanol/water *E. purpurea* extract exerted a significant IL-2 inhibitory effect at $50 \mu\text{g}$ plant material/mL (Fig. 2), the concentrations of total alkylamides and caffeic acid derivatives in each *E. purpurea* extract at this concentration were calculated (Fig. 3) based on quantities of these constituents in the undiluted extracts. Concentrations of caftaric acid, cichoric acid, and chlorogenic acid in the $50 \mu\text{g/mL}$ 95:5 ethanol/water extract were 15.0, 24.4, and 7.5 ng/mL , respectively, totaling $0.05 \mu\text{g/mL}$ caffeic acid derivatives. Concentrations of the alkylamides undeca-2(Z),4(E)-diene-8,10-diynoic acid isobutylamide and dodeca-2(E),4(E),8(Z),10(E/Z) tetraenoic acid isobutylamide were 0.05 and $1.76 \mu\text{g/mL}$, respectively, giving a total of $1.81 \mu\text{g/mL}$ alkylamides in this extract. Total alkylamide concentration in the 75:25 ethanol/water extract was $0.42 \mu\text{g/mL}$. The total caffeic acid derivative content was highest in the 50:50 ethanol/water extract ($0.81 \mu\text{g/mL}$).

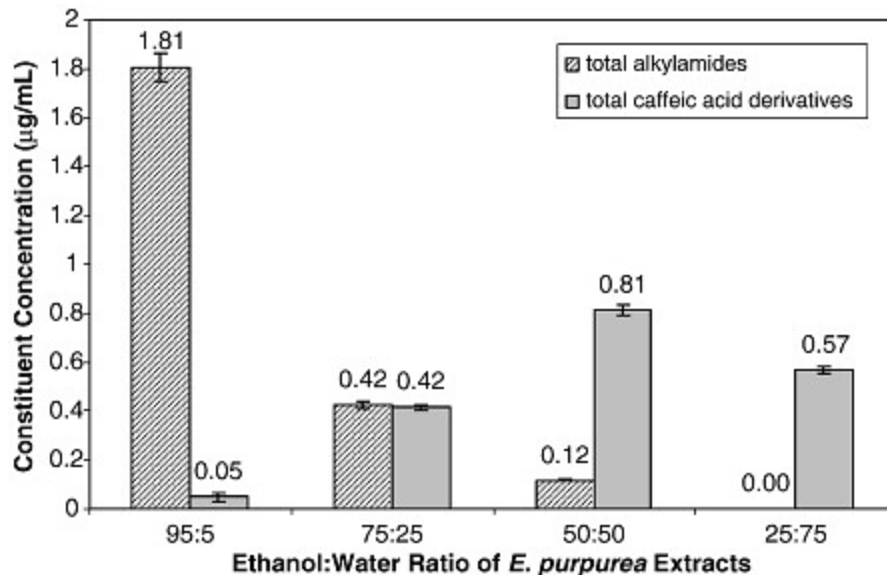
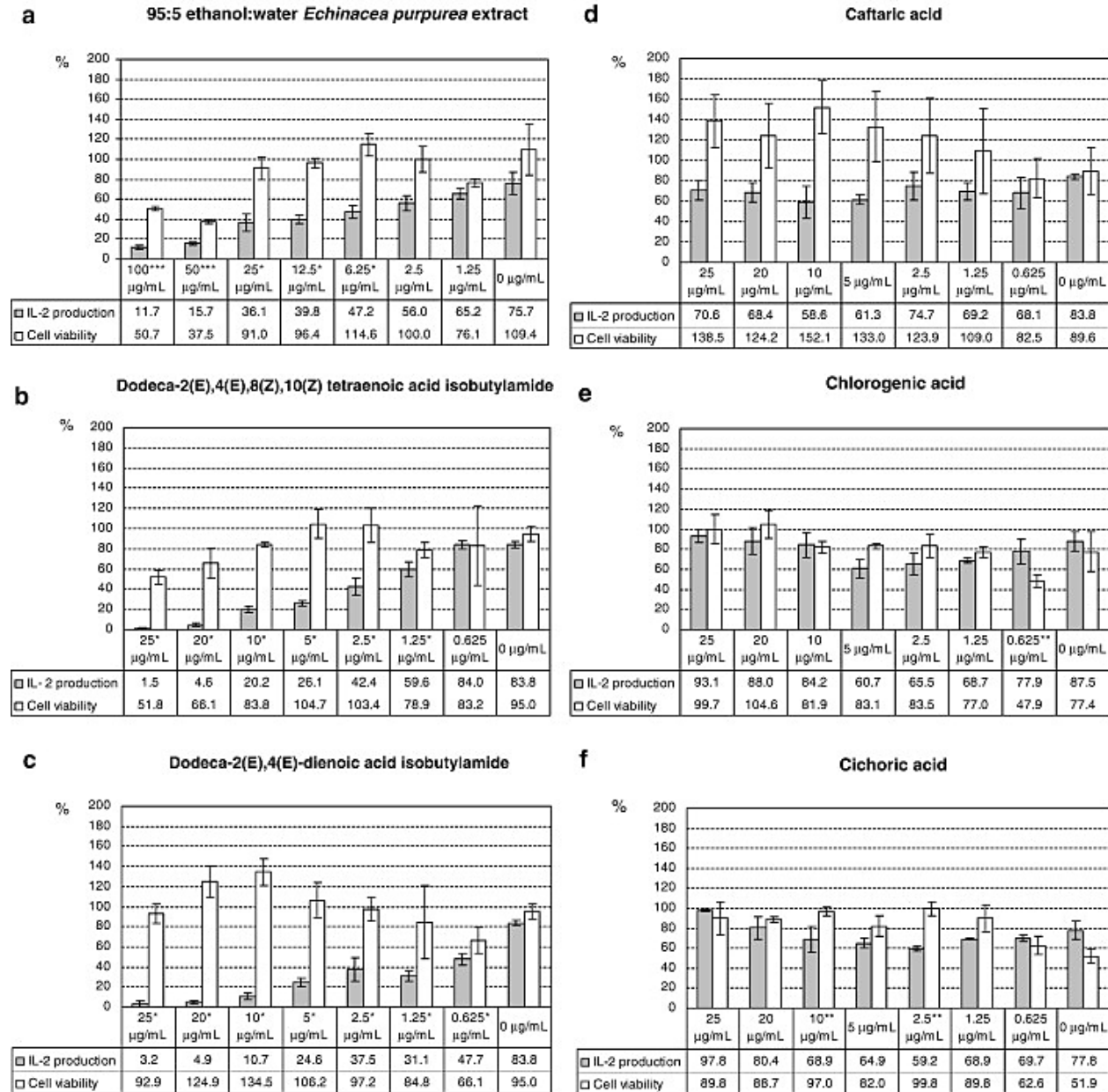


Fig. 3. Total alkylamide and caffeic acid derivative concentrations in *E. purpurea* extracts at 50 µg plant material/mL dilution. Concentrations were calculated based on the data obtained from quantities of constituents present in undiluted extracts as determined by HPLC/ESI-MS.

3.4. *Echinacea* alkylamides suppressed IL-2 production

We devised the simultaneous IL-2 production/cell viability assay to measure percent change in IL-2 production and cell viability relative to vehicle control following PHA/PMA stimulation of Jurkat T cells with or without *E. purpurea* extracts or chemical constituents thereof (Fig. 4a–f). The 95:5 ethanol/water *E. purpurea* extract dose-dependently suppressed IL-2 (Fig. 4a). At dilutions of 50 and 100 µg plant material/mL, this extract was also cytotoxic to the cells; the decreases in both IL-2 production and cell viability at these extract concentrations were statistically significant compared to vehicle control. However, at concentrations between 6.25 and 25 µg/mL, a statistically significant difference was observed in the IL-2 suppressive effect of this extract compared to vehicle ($p < 0.003$), but not in change in cell viability ($p > 0.219$).



Significant difference compared to vehicle: $p < 0.01$

- * = Statistically significant change in IL-2 production
- ** = Statistically significant change in cell viability
- *** = Statistically significant change in IL-2 production and cell viability

Fig. 4. Percent changes of IL-2 production and viable cell number relative to stimuli alone control. Cells were stimulated by PHA/PMA in the presence of vehicle or increasing concentrations of *Echinacea* 95:5 ethanol/water extract (a), or standard isolates (b–f). Percent change in IL-2 production or viable cell number relative to stimuli alone control is shown. Vehicle control is shown as 0 µg/mL. One-way ANOVA was performed on IL-2 change or viable cell number change with 95:5 extract or each constituent as a factor and each concentration as a different treatment. The treatment means ($N = 3$ independent wells) were

compared to vehicle control using Tukey's Honestly Significant Difference (HSD) post hoc analysis (two-tail, alpha level 0.01).

The effects of two standards of alkylamides, compounds which were present in the *E. purpurea* extracts with high ethanol content, were evaluated. Cells were treated with dodeca-2(*E*),4(*E*),8(*Z*),10(*Z*)-tetraenoic acid isobutylamide (Fig. 4b) or dodeca-2(*E*),4(*E*)-dienoic acid isobutylamide (Fig. 4c) at 0.625 to 25 µg/mL, which bracketed the concentration range of alkylamides present in the *E. purpurea* extract at test concentrations on cells. Both alkylamides showed a consistent and statistically significant dose-dependent IL-2 suppressive effect compared to vehicle ($p < 0.001$), while the slight changes in cell viability induced by these agents compared to the vehicle control were neither consistent nor statistically significant ($p > 0.08$).

3.5. *Echinacea* caffeic acid derivatives did not suppress IL-2 production

The effects of caffeic acid derivatives at concentrations relevant to the extract concentration were also evaluated. No caffeic acid derivative significantly altered IL-2 production at any concentration evaluated (Fig. 4d–f). However, the Tukey's HSD test showed a statistically significant decrease in cell viability caused by chlorogenic acid at the 0.625 µg/mL concentration ($p < 0.008$) (Fig. 4e). Cichoric acid showed a statistically significant proliferative effect on Jurkat cells at 2.5 ($p < 0.004$) and 10 µg/mL ($p < 0.006$) (Fig. 4f).

4. Discussion

Alkylamides were found at highest concentration in arial 95:5 ethanol/water *E. purpurea* preparations, and correlated with dose-dependent inhibition of IL-2 production by PHA/PMA-stimulated Jurkat T cells. This is the first report of an inhibitory effect of *Echinacea* alkylamides on production of IL-2, an important factor involved in expansion of clonal T cell populations in response to infection. By simultaneous assay of IL-2 production and cell viability, *Echinacea* alkylamide isolates were shown to significantly inhibit IL-2 but not cell viability. Studies to determine if the reported suppression is specific to IL-2 or whether alkylamides are more generally inhibiting to cytokine secretion are ongoing. One site of action of alkylamides has been identified as the cannabinoid 2 receptor (CB2) on monocytes/macrophages^[5]. Jurkat T cells express CB2^[22], so experiments to determine if the IL-2 suppressive effect is dependent on alkylamide binding to the CB2 receptor are warranted. Determination of the mechanism of alkylamide-induced IL-2 suppression is expected to contribute to the identification of appropriate and effective uses of *Echinacea*.

The physiological relevance of these results depends on a number of factors, including alkylamide pharmacokinetics and combination effects of different alkylamides and other constituents. Alkylamides do appear to be capable of reaching T cells in vivo. Alkylamides were detected in human serum at 17.4 ng/mL only 30 min after a single oral dose of *E. angustifolia* tincture^[23]; at 44 ng/mL after 1 h of a single dose of *E. purpurea* tincture^[24]; and at

336 ± 131 ng/mL within 20 min after ingestion of an ethanolic extract of *E. purpurea* and *E. angustifolia* in tablet form^[25]. The concentrations found in this last study are only two- to four-fold lower than the IL-2 suppressive alkylamide concentrations in this study. Thus, the hypothesis that alkylamide-induced IL-2 suppression modulates immune responsiveness in vivo warrants future testing.

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